

# Connective Tissue Alterations in the Skin of Ultraviolet Irradiated Hairless Mice

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Connective tissue alterations were induced in hairless mouse skin by ultraviolet (UV) irradiation. Hairless mice were irradiated three times a week for 10 weeks with sunlamps (UVA and UVB) and the skin was examined using immunochemical and biochemical techniques. Indirect immunofluorescence was performed with antibodies directed against elastin, microfibrillar proteins, and fibronectin. Increased fluorescence was observed in the actinically damaged skin for elastin, microfibrillar proteins, and fibronectin. The elastic fiber components, elastin and microfibrillar proteins, were then isolated and quantified. Control skin contained approxi-

mately 0.1% by dry weight of elastic fiber components, whereas actinically damaged skin contained 0.2% by dry weight. These data are consistent with previous observations of elastic fiber hyperplasia in UV irradiated mice. In addition, irradiated mouse skin contained 1.12 mg of extracted fibronectin per gram wet weight as compared with 0.59 mg in control skin. Irradiated mouse skin contained increased quantities of hyaluronic acid and chondroitin sulfate (uronic acid content). These studies further support the validity of the UV irradiated hairless mouse as a model of human dermal photoaging. *J Invest Dermatol* 91:158-161, 1988

**E**xcessive exposure of human skin to sunlight leads to various changes in the dermis [2]. Histologic studies [3,4] on actinically damaged skin have shown that the connective tissue components of the skin [5] are altered (i.e., accumulation of an elastotic material, and increased staining for glycosaminoglycans). Immunochemical studies [6,7] on human solar elastosis revealed that the elastotic material is composed primarily of elastin and microfibrillar proteins that codistribute with fibronectin (a glycoprotein component of the connective tissue). Collagen is the major fibrillar component of normal skin (70%-80% of the dry weight), whereas elastin and glycosaminoglycans are present in only minor amounts (2% and 0.1%, respectively). The hexosamine levels (glycosaminoglycan concentration) in the sun-damaged skin are significantly greater than that assayed in unexposed skin [8]. In contrast, the hydroxyproline content (measure of collagen) is significantly lower in the sun-damaged skin [9].

A major difficulty in analyzing the biochemical changes in the connective tissue components of human actinically damaged skin is the limited availability of tissue for study. As a result, haired [10] and hairless [11-15] mice models for photodamage have been developed in which dermal connective tissue alterations are induced by

ultraviolet radiation. These changes include increased amounts of thickened elastic fibers and glycosaminoglycans and a loss of collagen. Johnston et al [16] described certain biochemical changes in the dermis of these irradiated mice including increased desmosine content (indication of elastin concentration) and decreased prolyl hydroxylase activity.

The aim of this study was to use immunochemical and biochemical techniques to evaluate the alterations in the elastic fibers and glycosaminoglycans observed by histologic staining in UV irradiated hairless mouse dermis. Frozen thin sections of irradiated and non-irradiated mice skins were reacted with antibodies directed against elastin, microfibrillar proteins, and fibronectin. The elastic fiber components, elastin and microfibrillar proteins, were isolated and quantified. The amount of fibronectin present in extracts of the skin was determined by a specific Enzyme-linked Immunosorbent Assay. Glycosaminoglycans were purified and the uronic acid content determined. These ultraviolet induced changes were compared with connective tissue alterations described in human actinically damaged skin.

## MATERIALS AND METHODS

**Animal Treatment** Hairless albino mice (SKH/hr-1 strain), 10 weeks old, were irradiated by a bank of six Westinghouse FS20 sunlamps placed 45 cm above the backs of the mice. Exposure times, three times a week, were 10 min a day for the first 4 weeks (0.1 J/cm<sup>2</sup> for UVA and UVB). These times were then increased by 5 min every 2 weeks until at 10 weeks the exposure times were 30 min (0.3 J/cm<sup>2</sup> for UVA and UVB). Irradiance, as measured with an IL 700A Research Radiometer (International Light, Inc., Newburyport, MA), was generally 185 uW cm<sup>-2</sup>. A group of age-matched mice that were not irradiated served as controls. After 10 weeks of irradiation, animals from the control and irradiated groups were killed by cervical dislocation and strips of dorsal skin were removed. Animals were irradiated in groups of 10-20 mice throughout these studies. A total of approximately eighty mice (irradiated and control) were used.

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A preliminary report of this study was presented at the Annual Meeting of the Society for Investigative Dermatology, San Diego, CA, 1987 [1].

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### Abbreviations:

- CNBr: cyanogen bromide
- ELISA: Enzyme-linked Immunosorbent Assay
- PBS: phosphate-buffered saline
- SDS: sodium dodecyl sulfate
- UV: ultraviolet

**Indirect Immunofluorescence** Frozen sections of mouse skin, about 8  $\mu$  thick, were defatted in ethyl alcohol:ether (1:1) followed by 70% alcohol. The specimens were digested for 30 min with hyaluronidase (2 mg/ml in PBS) and then successively treated for 30 min at 37°C in a humidified chamber with the specific antibody, washed with PBS, and treated for 30 min with goat antirabbit IgG conjugated with fluorescein isothiocyanate. The sections were then washed with PBS and mounted in PBS:glycerol (1:9, v/v) containing 0.3M triethylenediamine to reduce the fading of the fluorescence. Samples were examined with a Nikon Fluophot Research Microscope. Controls consisted of IgG prepared from the sera of non-immunized rabbits. Antibodies against bovine elastin were obtained from Elastin Products Co. (Pacific, MO) and antibodies against bovine microfibrillar proteins were prepared as previously described [17]. Rabbit antibovine fibronectin was obtained from Calbiochem (LaJolla, CA).

**Separation of Connective Tissue Components** Dorsal skins from ten irradiated and ten control mice were diced into small pieces and homogenized using a Virtis (40,000 rpm, 10 min, on ice) in 20 ml of PBS containing proteolytic inhibitors (0.02% sodium azide, 2 mM phenylmethylsulfonyl fluoride, 1 mM N-ethyl maleimide, and 10  $\mu$ g/ml aprotinin). The suspension was centrifuged (5000 rpm, 10 min, 0–4°C), the supernatant decanted, and the pellet treated with 100 ml of chloroform-methanol (1:1). The dried pellets were then extracted sequentially (in the presence of proteolytic inhibitors) with 1M NaCl/0.05M Tris (pH 7.5), 0.5N acetic acid, 4.0M guanidine HCl/0.05M Tris (pH 7.5), and 9.5M urea/0.1M mercaptoethanol/0.005M Tris (pH 8.0). The insoluble fraction was then digested with cyanogen bromide (CNBr) as follows: 10 mg of the pellet was suspended in 3 ml of formic acid containing 1 gm of CNBr. The mixture was stirred at room temperature under nitrogen for 5 h, diluted with water, and lyophilized. The residue was resuspended in 0.5N acetic acid and centrifuged at 12,000 rpm for 30 min (0–4°C). The CNBr insoluble fraction was washed twice with 0.5N acetic acid, lyophilized, and subjected to a Lansing procedure [18] with hot alkali (0.1N NaOH, 98°C, 45 min). The suspension was centrifuged and the pellet washed three times with water before lyophilization.

**Fibronectin Analysis** Skins (approximately 1 gm wet weight) from irradiated ( $n = 4$ ) and control ( $n = 4$ ) animals were individually minced into small pieces, suspended in 20 ml of 0.05M Tris (pH 7.5) containing proteolytic inhibitors, and homogenized at 40,000 rpm for 10 min (Virtis). Sodium dodecyl sulfate (SDS) and mercaptoethanol were then added to a final concentration of 1% and 0.33M, respectively. The samples were extracted overnight at room temperature with stirring and centrifuged at 5,000 rpm for 30 min (room temperature). The supernatants were dialyzed extensively against water and lyophilized. The samples were reconstituted in water to 2 ml and analyzed in duplicate for extracted fibronectin [19] by a specific Enzyme-linked Immunosorbent Assay (ELISA) utilizing rabbit antibovine fibronectin.

**Glycosaminoglycan Analysis** Three groups ( $n = 3$ ) of irradiated (5 mice per group) and control (5 mice per group) skins were analyzed [20] by the method of Schiller et al [21] as modified by Mier and Wood [22]. Defatted dried skins were digested with papain and trypsin and treated with 0.5N NaOH. Protein was then removed with 10% trichloroacetic acid. The glycosaminoglycans were precipitated with cetylpyridinium chloride and subjected to differential salt extraction with 0.4 M (for hyaluronic acid), 1.2 M (for chondroitin sulfate), and 2.1 M (for heparin) NaCl solutions. Uronic acid was determined in each fraction by the carbazole method [23].

**Amino Acid Analysis** Lyophilized samples were hydrolyzed in constant boiling HCl (5.7N) for 24 h at 107°C. Amino acid residues were derivatized with 9-fluorenylmethyl chloroformate and then resolved by reverse phase high-pressure liquid chromatography using a 250  $\times$  4.6 mm Spherisorb ODS-2 column [24]. The separation was carried out by gradient elution as follows: 0–15 min, 60% acetic acid (0.3%, pH 4.2)/15% water/ 25% acetonitrile; 15–

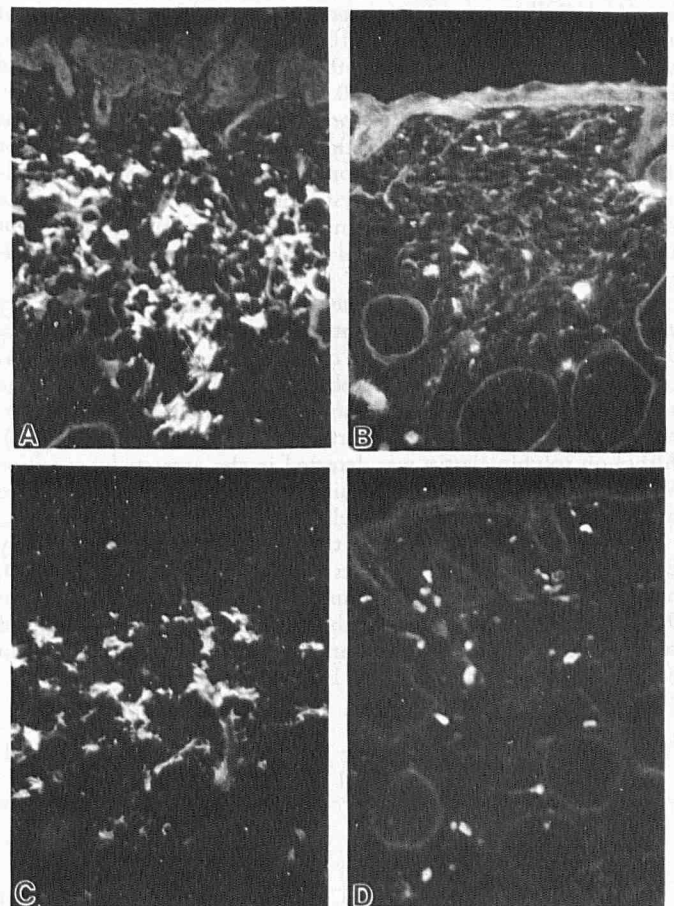
75 min, 60/15/25 to 28/7/65. The flow rate was maintained at 1 ml/min and the fluorescence was monitored (excitation 260 nm, emission 350 nm cut off filter) using a Kratos FS970 Spectrofluorometer.

## RESULTS

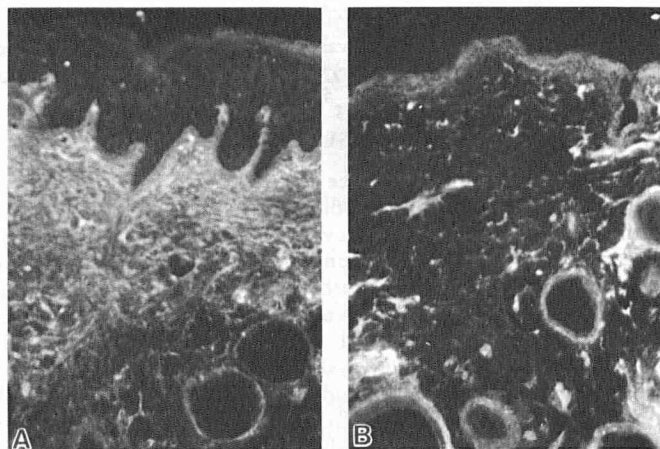
**Indirect Immunofluorescence Studies** Indirect immunofluorescence techniques with antibodies directed against elastin, microfibrillar proteins, and fibronectin were used to detect modulations in these connective tissue components. The results obtained using antibodies directed against the elastic fiber components, elastin, and microfibrillar proteins, are shown in Fig 1. Increased fluorescence was seen with the elastin (Fig 1A, B) and microfibrillar proteins (Fig 1C, D) antisera in the irradiated skin as compared with the control skin. Sections from irradiated and control skins were negative following treatment with sera from nonimmunized rabbits (not shown). The results obtained with fibronectin antisera on irradiated and control skins are shown in Fig 2. Sections reacted with fibronectin antisera from irradiated skin show more intense fluorescence than seen in control skin.

The increased fluorescence could correspond to an increased amount of antigen or to an alteration in the binding. Therefore, the amount of antigen present was analyzed.

**Biochemical Studies** A purification scheme was devised to separate the elastic fiber components from collagen and other proteins. The yields obtained are given in Table I and the values represent the average from two separate experiments. The urea/mercaptoethanol insoluble fraction was composed of collagen, elastin, and micro-



**Figure 1.** Indirect immunofluorescence of irradiated and control mice skins stained with antibodies directed against bovine elastin (A, B) and bovine microfibrillar proteins (C, D). Increased staining is observed for both antibodies in the irradiated skin (A, C) over that seen in control skin (B, D) (160X).



**Figure 2.** Indirect immunofluorescence of irradiated and control mice skins stained with antibodies directed against bovine fibronectin (A, B). The epidermis is acanthotic and the stratum corneum is thickened in the irradiated mouse skin (A) as compared with control skin (B). The fluorescence obtained with the fibronectin antibody appears to be more intense in the irradiated skin (160X).

fibrillar proteins. This fraction was then treated with CNBr to remove the collagen component. Next, the CNBr insoluble fraction was subjected to hot alkali hydrolysis to further purify the elastic fiber components. The isolation of elastin was monitored by the ratio of hydroxyproline to proline determined in the various fractions. Collagen contains approximately 100 residues each of hydroxyproline and proline per 1000 amino acid residues, whereas elastin contains approximately 10 residues of hydroxyproline and 100 residues of proline per 1000 amino acid residues. Therefore, collagen has a ratio of hydroxyproline to proline of 1.0, whereas elastin has a ratio of 0.1. The ratios of 0.24 and 0.34 of the CNBr insoluble fraction showed that some collagen remained in the pellet. Following hot alkali hydrolysis, the ratios fell to an acceptable value of 0.1. In addition, the amino acid composition of the hot alkali insoluble fraction indicated that a mixture of elastin and microfibrillar proteins was present.

Control mouse skin contained approximately 0.1% by dry weight of elastic fiber components. In irradiated skin, the quantity doubled to 0.2%. These data are consistent with previous observations [14] of elastic fiber hyperplasia in UV irradiated mice. The amount of elastic fiber components in normal mouse skin was, however, only a fraction of that reported for normal human skin (2%). Although soluble elastin was detected in the extracts by sensitive Western immunoblotting techniques, the amount could not be measured by specific ELISA for elastin.

Fibronectin was quantified in the SDS extract of intact skin by ELISA. Irradiated dermal extracts contained  $1.12 \text{ mg} \pm 0.08$  (SD,  $n = 4$ ) of fibronectin per gram wet weight as opposed to  $0.59 \text{ mg} \pm 0.14$  (SD,  $n = 4$ ) of fibronectin per gram wet weight of control skin. These results document the increase in fibronectin previously visualized by indirect immunofluorescence.

**Table I.** Purification of Elastic Fiber Components in Irradiated and Control Mice Skins

	Dry Weight (mg)		Ratio of Hydroxyproline to Proline	
	Irradiated	Control	Irradiated	Control
Defatted tissue	1060	1050	0.63	0.75
CNBr insoluble	4.87	2.27	0.24	0.34
Hot alkali insoluble	2.00	0.86	0.11	0.10
(Yield of elastic fiber components)	(0.2%)	(0.1%)		

The amount of fractionated glycosaminoglycans in irradiated and control mice skins is given in Table II. Previous studies have identified the major fractions as hyaluronic acid (0.4M NaCl extract), chondroitin sulfate (1.2M NaCl extract), and heparin (2.1M NaCl extract). The majority of the glycosaminoglycans of mouse skin was found in the 0.4M and 1.2M NaCl extracts. Irradiated mouse skin showed an increase in the uronic acid content of the hyaluronic acid and chondroitin sulfate fractions. Data from three distinct preparations of irradiated and control skins are given in Table II. These results confirm previous qualitative histochemical studies [14].

## DISCUSSION

Qualitative histologic and limited quantitative biochemical studies have been done to evaluate photodamage in the hairless mice. Histologic studies have shown an increase in both the size and number of elastic fibers and an increased staining for glycosaminoglycans following prolonged exposure to ultraviolet light. Johnston et al [16] have shown that short exposures of hairless mice to ultraviolet light reduced prolyl hydroxylase activity (measure of collagen synthesis). Plastow et al [25] determined that irradiated skin contained elevated amounts of type III collagen. Studies on elastin alteration have been limited to the determination of desmosine levels [16] following irradiation. Analyses of fibronectin and glycosaminoglycan levels were not performed.

This publication reports both immunochemical and biochemical studies on irradiated and control skins. An increase in staining for elastin, microfibrillar proteins, and fibronectin was observed by indirect immunofluorescence. Elastic fiber components (elastin and microfibrillar proteins) and extracted fibronectin were found to increase twofold using biochemical techniques following irradiation. This accumulation of elastin, microfibrillar proteins, and fibronectin was previously described in sun-damaged human skin, and the interaction of fibronectin with elastic tissue has also been noted in pseudoxanthoma elasticum [26]. These UV-induced changes are consistent with those observed in human actinically damaged skin. However, the elastin content of the mouse skin was found to be approximately 5%–10% of that found in human skin.

The changes in the glycosaminoglycan content are similar in sun-damaged human skin and irradiated hairless mouse skin. Elevated hexosamine content has been found in actinically damaged human skin [8]. The UV-irradiated hairless mouse dermis contained greater quantities of uronic acid (i.e., hyaluronic acid and chondroitin sulfate) than control skin.

These studies help to confirm the validity of the UV irradiated hairless mouse as a model for human dermal photoaging. Although the magnitude of the connective tissue alterations may differ in the two systems the pattern of the observed changes remains similar. This model can be used to study the effect of prolonged solar exposure on the biochemistry of elastin. Significant quantities of elastin could be isolated from the skins of UV-irradiated mice and any structural changes delineated. The hairless mouse is therefore a viable model with which to study the biochemical mechanisms that govern the UV-induced connective tissue alterations.

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**Table II.** Glycosaminoglycan Content in Irradiated and Control Mice Skins

	ug uronic acid/g dry weight (mean $\pm$ SD)		
	0.4 M NaCl	1.2M NaCl	2.1M NaCl
Irradiated Mice (n = 3)	$107.0 \pm 9.7$	$130.0 \pm 14.7$	$26.0 \pm 2.0$
Control Mice (n = 3)	$48.3 \pm 10.1$	$47.0 \pm 8.7$	$18.3 \pm 1.5$



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## CUTANEOUS LYMPHOMA

An international symposium sponsored by the European Society for Dermatological Research will be held October 28-30 at the Ermitage Hotel, Copenhagen, Denmark. For further information contact: Gunhild R. Lange Wantzin, M.D., Bispebjerg Hospital, Department of Dermatology, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark. Telephone: 0045 1 81 12 50 (ext. 3170).